HUMAN NEUTROPHILS ACTIVATED VIA TLR8 PROMOTE Th17 POLARIZATION THROUGH IL-23

Running title: Interleukin-23 expression by human neutrophils

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Summary sentence: This study shows that human neutrophils incubated with TLR8 agonists produce interleukin-23, with which promote a Th17 polarization from naïve T cells

Abbreviations

CCL	chemokine CC motif ligand
CD	cluster of differentiation
ChIP	Chromatin immunoprecipitation
ChIP-seq	ChIP followed by high throughput sequencing
ELISA	enzyme-linked immunosorbent assay
FPKM	fragments per kilobase of transcript per million mapped reads
G-CSF	granulocyte colony-stimulating factor
GAPDH	glyceraldehyde phosphate dehydrogenase
GM-CSF	granulocyte-macrophage colony-stimulating factor
HOMER	Hypergeometric Optimization of Motif EnRichment
IL	Interleukin
mRNA	messenger RNA
LPS	lipopolysaccharide
PAMPs	pathogen associated molecular patterns
PBMCs	peripheral blood mononuclear cells
PRR	pattern recognition receptor
RT-qPCR	reverse transcription quantitative Polymerase Chain Reaction
Th	T helper
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TSS	Transcriptional start site
UCB	Umbilical cord blood

Abstract

Human neutrophils contribute to the regulation of inflammation via the generation of a range of cytokines that affect all elements of the immune system. Here, we investigated their ability to express some of the members of the IL-12 family after incubation with TLR8 agonists. Highly pure human neutrophils were thus incubated for up to 48 h with or without, R848, or other TLR8 agonists, to then measure the expression levels of transcripts and proteins for IL-12 family member subunits by RNA-seq, RT-qPCR and ELISA. We show a TLR8-mediated inducible expression of IL12B and IL23A, but not IL12A, mRNA, which occurs via chromatin remodelling (as assessed by ChIP-seq), and subsequent production of IL-23 and IL-12B, but no IL-12, proteins. Induction of IL-23 requires endogenous TNFα, as both mRNA and protein levels were blocked in TLR8-activated neutrophils *via* a TNFα-neutralizing antibody. We also show that supernatants from TLR8-activated neutrophils, but not autologous monocytes, induce the differentiation of Th17 cells from naïve T cells in an IL-23-dependent fashion. This study unequivocally demonstrates that highly pure human neutrophils express and produce IL-23, further supporting the key roles played by these cells in the important IL-17/IL-23 network and Th17 responses.

INTRODUCTION

Neutrophils are currently recognized as potential sources of cytokines, including chemokines and growth factors[1]. Neutrophil-derived cytokines not only regulate inflammation and immunity, but also orchestrate a variety of physiological processes such as haematopoiesis, angiogenesis and fibrogenesis, as well as pathological conditions such as infectious, inflammatory, autoimmune or neoplastic diseases[2-4]. Circulating blood neutrophils from healthy individuals do not normally express cytokines, but can generate them in response to stimulus-specific environmental signals[1]. Many ligands can activate cytokine expression by human neutrophils, for instance microbial factors such as pathogen-associated molecular patterns (PAMPs) binding to pattern recognition receptors (PRR), including Toll-like receptors (TLRs)[5], (RIG-I)[6, 7] and DNA sensors[7-9], or host-generated cytokines. In addition, neutrophil-derived factors can themselves enhance/generate additional cytokine expression via autocrine feedback loops[10-12].

We recently identified R848 and CL075 as very powerful agonists able to trigger a remarkable extracellular production of cytokines, including TNFα, IL-6, G-CSF and CCL23[11, 13, 14]. R848 and CL075 are synthetic compounds that in human neutrophils specifically act via TLR8, since TLR7, their other receptor, is absent and not inducible following cell activation [5, 11, 14]. Interestingly, by investigating the molecular pathways leading to the expression of IL-6 mRNA – an IκBζ-dependent gene -, we identified a previously undescribed mechanism of cytokine induction in human neutrophils, resulting from chromatin remodelling[11]. Specifically, we observed that the induction of IL-6 transcription by R848 depended on an inducible remodelling of chromatin at the IL-6 genomic locus, turning it from an “inactive” to an “active” configuration[11]. We also observed that, among the IκBζ-dependent

genes, transcripts for IL-12B (corresponding to the p40 subunit of the IL-12 cytokine family) were greatly induced in TLR8-treated neutrophils[11]. However, we did not further investigate whether IL12B mRNA was converted into protein, and/or whether other members of the IL-12 cytokine family to which p40 associates are expressed/produced by TLR8-activated neutrophils. IL12B is a β-chain of the IL-12 family of heterodimeric cytokines that can assemble with some α-chains of the same family, such as IL23A (the p19 subunit), and IL12A (the p35 subunit), to generate the heterodimers IL-12 (IL12A plus IL12B, which form the p70 complex) and IL-23 (IL23A plus IL12B)[15]. In addition, human IL-12B can also form homodimers that bind to the IL-12 receptor but these do not mediate any biologic activity[16].

Because of the critical roles of the IL-12 family members in innate and adaptive immunity[17, 18], we specifically investigated whether human neutrophils activated via TLR8 express/produce IL-12 and/or IL-23. We report that TLR8-treated neutrophils, unlike circulating blood neutrophils, express and produce IL-23, but not IL-12. We also report that supernatants from TLR8-activated neutrophils promote the differentiation of Th17 cells from naïve T cells, a finding that adds a new dimension to the ability of these cells to regulate immune functions during infections and inflammation.

MATERIALS AND METHODS

Cell Purification and culture. Highly-purified neutrophils were isolated from the venous blood or from buffy coats from healthy individuals using a combined method consisting of Ficoll-Paque gradient centrifugation, dextran or Hetasep (StemCell Technologies, Vancouver, Canada) sedimentation of granulocytes and hypotonic lysis of erythrocytes, followed by removal of contaminating immune cells using the EasySep neutrophil enrichment kit (StemCell Technologies, Vancouver, Canada) as described previously[19, 20]. This procedure yields neutrophils of approximately 99.7% purity. Human monocytes were isolated from PBMCs, after Ficoll-Paque gradient centrifugation, by anti-CD14 microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) to reach > 98 % purity. Neutrophils and monocytes were then suspended at 5×10^6/ml and 2.5×10^6/ml respectively, in RPMI 1640 medium containing 10 % (< 0.5 EU/ml endotoxin fetal bovine serum (BioWhittaker-Lonza, Basel, Switzerland). Cells were incubated for periods of up to 48 h (as indicated in the text) in the absence (control) or presence of 0.25-10 μM R848 (InvivoGen, San Diego, CA, USA), 0.25-10 μM CL075 (InvivoGen), 0.25-10 μM VTX-2337 (Selleck Chem, Boston, MA, USA), 1 μg/ml LPS (ultrapure, *E. coli* 0111:B4 strain, InvivoGen), 10 ng/ml TNFα (R&D Systems, Minneapolis, MN, USA) or 10 μg/ml adalimumab (Humira, Abbott Biotechnology Limited, Barceloneta, Puerto Rico).

Cell viability. Cell viability was assessed by flow cytometry using Vybrant DyeCycleTM Violet (Thermo Fisher Scientific, Waltham, MA, USA) and SYTOX AADvanced (Thermo Fisher Scientific) stain, as previously described [14].

RNA isolation. After incubation as described in the text, neutrophils were pelleted by centrifugation, and total RNA was extracted with either Trizol or RNeasy mini kit (Qiagen, Venlo, Limburg, Netherlands). To completely remove any possible contaminating DNA, an on-column DNase digestion with the RNase-free DNase set (Qiagen) was performed during total RNA isolation.

RT-qPCR. Total RNA was reverse-transcribed into cDNA using Superscript III (Thermo Fisher Scientific) and random hexamer primers (Thermo Fisher Scientific). Transcript levels of individual genes were measured by RT-qPCR using specific primer pairs (Thermo Fisher Scientific or Bio-Rad, Hertfordshire, UK) listed in Table 1. Data were calculated by Q-Gene software (http://www.gene-quantification.de/download.html) and expressed as mean normalized expression units after RPL32 normalization.

RNA-seq. Prior to RNA-seq, total RNA was enriched for mRNA using poly(A) selection. Standard Illumina (San Diego, CA, USA) protocols were used to generate 50 bp single-end read libraries. In brief, mRNA was fragmented, reverse transcribed, adapted with sequencing primers and sample barcodes, size selected, and PCR-enriched. Libraries were sequenced on the Illumina HiSeq 2000 platform. Reads were mapped to the reference human genome (hg19) using TopHat version 2.0.14 and Bowtie 2 version 2. Gene expression values [Fragments Per Kilobase of transcript per Million mapped reads (FPKM)] were calculated using Cufflinks version 2.02. A minimum FPKM threshold of expression of ≥ 0.3 was applied to the expression data to minimize the risk of including false positives against discarding true positives from the datasets.

Immunoblotting experiments. Total proteins from neutrophils and monocytes were recovered from protein-rich flow-through solutions obtained after the first centrifugation step of the RNeasy mini kit procedure (Qiagen, used for total RNA extraction), as previously described [11]. Proteins were then immunoblotted by standard procedures using the anti-human IRF8 pAbs (kindly provided by Prof. G Natoli from Humanitas University, Milan), and anti-human β-actin mAbs (A5060 from Sigma, Saint Louis, MO, USA). Blotted proteins were detected by the Odyssey infrared imaging system (LI-COR Biosciences, Lincoln, NE, USA) [11].

Chromatin immunoprecipitation (ChIP) Assays. Chromatin immunoprecipitation experiments were performed exactly as previously described [11]. Briefly, nuclear extracts from formaldehyde-fixed 2.5-10 × 10^6 neutrophils were immunoprecipitated using 4 μl anti-PU.1 (sc-352) or anti-H3K27Ac (ab4729) pAbs (Abcam, Cambridge, United Kingdom). Coimmunoprecipitated material was subjected to qPCR analysis using the specific primers listed in Table 2 (purchased from Thermo Fisher Scientific). Data from qPCR are expressed as percentage over input DNA and are displayed as mean ± SEM.

ChIP-seq. Purified DNA from PU.1 and H3K27Ac ChIP assays (performed as described in the previous paragraph) was adapter-ligated and PCR-amplified for sequencing on HiSeq2000 platform (Illumina, Cambridge, UK) using TruSeq DNA Library Prep Kit (Illumina). After sequencing, reads were quality-filtered according to the Illumina pipeline. Single end (51 bp) reads were then mapped to the human genome (Genome Reference Consortium GRCh37, Feb/2009) using BOWTIE v1.0.0.

Only reads with no more than two mismatches (when compared to the reference genome) were converted to tag directories using HOMER's module known as "makeTagDirectory," and then converted to BedGraph format using HOMER's module known as "makeUCSCfile," to be finally normalized to 10^7 total tag counts. ChIP-seq signals were visualized using Integrative Genomics Viewer.

Cytokine production. Cytokine concentrations in cell-free supernatants were measured by commercially available ELISA kits, specific for human: IL-1β (eBioscience, San Diego, CA, USA), IL-23 (Mabtech, Nacka Strand, Sweden), IL-23A/IL-23p19 (Abcam, Cambridge, United Kingdom), TGFβ1 (R&D Systems), IL-6, IL-12/IL-12p70 and IL-12B/IL-12p40 (Mabtech). Lower detection limits of these ELISA were: 4 pg/ml for IL-1β, 4 pg/ml for IL-23, 20 pg/ml for IL-23A, 31 pg/ml for TGFβ1, 8 pg/ml for IL-6, 6 pg/ml for IL-12 and 10 pg/ml for IL-12B.

Functional Assays. Isolation of $CD4^+$ T cells from PBMCs of umbilical cord blood (UCB) was performed by using the CD4 isolation kit II (Miltenyi Biotec). After washing, cells were separated by immunomagnetic cell sorting (Miltenyi Biotec). Purified UCB-derived $CD4^+$ T cells were incubated for 1 week with 5 μg/ml of anti-CD3 plus 5 μg/ml anti-CD28 mAbs, in the absence or the presence of either 10 ng/ml IL-1β plus 20 ng/ml IL-23 (R&D Systems), or the indicated conditioned media from neutrophils and monocytes. In some experiments, anti-human IL-23 p19 antibodies (R&D Systems) were used in culture at the final concentration of 10 μg/ml. On day 7, T cells were stimulated with 10 ng/ml PMA plus 1 μM ionomycin for 6 h, the last 4 h in the presence of 5 μg/ml brefeldin A, and then analyzed for intracellular IL-17, in

association with CD161 membrane expression, by BD LSRII flow cytometry with FACS Diva software (BD Biosciences, Franklin Lakes, NJ, USA), as previously described [21, 22].

Statistical Analysis. Data are expressed as mean ± SEM. Statistical evaluation was performed by using, depending on the experimental data, Student's t test, 1-way ANOVA followed by Tukey's post hoc test or 2-way ANOVA followed by Bonferroni's post hoc test. P values < 0.05 were considered as statistically significant.

Study Approval. Human samples were obtained following informed, written, consent by healthy donors in accordance with the Declaration of Helsinki. This study was carried out in accordance with the recommendations of Committee on Research Ethics (CORE, University of Liverpool), Ethic Committee of the Azienda Ospedaliera Universitaria Integrata di Verona (Italy) and the Regional Committee on Human Experimentation (Florence, Italy).

RESULTS

Human neutrophils incubated with R848 and other TLR8 agonists are induced to express IL12B and IL23A, but not IL12A, mRNA.

In initial experiments, we incubated highly pure human neutrophils in the absence (control) and the presence of 5 μM R848 - in line with our previous studies [11, 13] - and then measured changes in global gene expression by RNA-seq at 7 h and 20 h. Under these experimental conditions, survival rate of neutrophils was higher than 75 % and 20 % after, respectively, 20 and 44 h of incubation [14]. Notably, in R848-treated neutrophils, mRNAs for IL23A and IL12B, the constituent components of IL-23[23], were expressed at remarkably high levels at both time-points (Fig. 1A, and data not shown). By contrast, mRNA levels of IL12A, the chain associating with IL12B to form IL-12 [24], were at, or below, the detection limit and cut-off threshold FPKM values (< 0.3) in both control and R848-treated neutrophils (Fig. 1A). We then performed kinetic experiments by RT-qPCR (Fig. 1B), which not only confirmed the RNA-seq data, but also indicated that the optimal incubation time with R848 to induce maximal expression of IL12B and IL23A mRNAs in human neutrophils is 12 h. Consistent with the RNA-seq data (Fig. 1A), IL12A remained substantially undetectable at all time-points investigated (Fig. 1B). Interestingly, induction of IL12B and IL23A mRNAs by R848 was only detected after 3 h of incubation (Fig. 1B), which, at least in the case of IL12B, is consistent with the necessity to preliminarily activate the synthesis of IκBζ[25]. Incubation of human neutrophils with other TLR8 ligands, such as 1 μM CL075[26] or 2 μM Motolimod/VTX-2337[27] showed similar results (data not shown). Collectively, these data demonstrate that highly pure human neutrophils incubated with R848 express the mRNAs encoding the chains composing IL-23, but not IL-12.

The *IL23A* and *IL12B* genomic loci of human neutrophils are characterized by latent enhancers

To identify and characterize, at a whole genome level, changes in the genomic regulatory regions of neutrophils following incubation with R848, we performed ChIP-seq experiments (NT *et al*, manuscript in preparation) for the genome-wide mapping of both histone 3 lysine 27 acetylation (H3K27Ac, marking active cis-regulatory elements) and PU.1 (a myeloid lineage determining TF). Based on the results shown in Fig. 1, we analyzed the levels of the H3K27Ac and PU.1 peaks at the *IL12A*, *IL12B* and *IL23A* genomic loci, to infer precise information of their chromatin status in neutrophils. As shown in Fig. 2A, no PU.1 or H3K27Ac peaks were detectable at the *IL12A* locus, regardless of the neutrophil-treatment with R848. This finding indicates an inactive genomic region, in line with the absence of *IL12A* transcription as shown in Fig. 1A and B, as well as Fig. 2A. By contrast, the levels of H3K27Ac and PU.1 at the genomic regulatory regions of both *IL12B* and *IL23A* were found in a latent state [28], being very low in untreated neutrophils (Fig. 2B and C), but dramatically increased following neutrophil incubation with R848. This was especially evident at the genomic regions located at 12, 29 and 31 kb from the *IL12B* TSS (Fig. 2B), and at 2.5 and 5.5 kb from the *IL23A* TSS (Fig 2 C). Altogether, data from these ChIP-seq experiments not only are in line with the results shown in Fig. 1, but also support the notion that, in human neutrophils, chromatin re-modelling regulates IL12B and IL23A mRNA induction by R848, similarly to that previously observed for IL-6 [11].

Neutrophils incubated with R848 produce IL-23, but not IL-12.

We then prepared cell-free supernatants harvested from neutrophils and (as control) autologous $CD14^+$ monocytes to measure the levels of IL-12B, IL-12 and IL-23 by specific ELISA kits (Fig. 3). In these experiments, cells were also incubated with 1 μg/ml ultrapure LPS, to compare its activity to that of R848. Neutrophils incubated for 20 h with R848 were found to produce and release both IL-23 and IL-12B, but not IL-12 (Fig. 3A), with IL-23 and IL-12B levels further increasing after 44 h incubation (Fig. 3A). A similar qualitative pattern of neutrophil-derived cytokines was observed in response to LPS, but at lower levels (Fig. 3A). Measurements of the IL-23A monomer in supernatants from unstimulated or LPS- and R848-stimulated neutrophils for 20 h (1.7 ± 0.6, 17.4 ± 6.1 and 23.6 ± 5.5 pg/ml, respectively) did not substantially differ from that of the IL-23 heterodimer. Additional experiments confirmed that 5 μM R848 represent the optimal concentrations to induce the production of IL-23 and IL-12B by neutrophils (data not shown), and that other TLR8 agonists, including 1 μM CL075[26] or 2 μM VTX-2337[27], also potently induce the release of these cytokines (data not shown). Not surprisingly [26, 29], $CD14^+$ monocytes incubated with R848 and LPS produced much higher amounts of both IL-23 and IL-12B than neutrophils (Fig. 3B). However, in contrast to neutrophils, activated $CD14^+$ monocytes also produced IL-12, particularly in response to R848 (Fig. 3B), confirming previous observations [26]. Accordingly, we found that monocytes do accumulate IL12A mRNA upon TLR8 activation (**data not shown**), consistent with similar observations made by other groups [30, 31]. We also observed that $CD14^+$ monocytes, unlike neutrophils (Fig. 3B), express high levels of IRF8, a transcription factor that, by interacting with IRF1 at the IL23A genomic locus, is essential for IL-12A transcription [32]. Altogether, these data show that human neutrophils treated with R848 produce IL-23, but not IL-12, protein.

3.5 Biological activity of neutrophil-derived supernatants

Because IL-23, along with IL-1β, is involved in promoting the differentiation of Th17 cells [33], we tested whether supernatants harvested from neutrophils and, for comparison, autologous $CD14^+$ monocytes, could induce umbilical cord blood (UCB)-derived *naïve* CD4 T cells to differentiate into Th17 cells [22]. For these experiments, UCB-derived *naïve* CD4 T cells were polyclonally stimulated with anti-CD3 and anti-CD28 mAbs and cultured in medium in the absence, or the presence, of IL-1β plus IL-23 as a positive control (Fig. 4A, top panels), as well as in supernatants derived from either neutrophils or $CD14^+$ monocytes incubated for 24 h with or without R848 (Fig. 4A, bottom panels). Previous measurement of IL-1β in supernatants from R848-treated neutrophils and monocytes revealed concentrations of 11.6 pg/ml and 1.3 ng/ml, respectively. We also found IL-6 levels corresponding to 180 pg/ml and 200 ng/ml in R848-stimulated neutrophils and monocytes, respectively, while TGFβ1 levels were below the threshold limit of the ELISA used. As shown in Fig. 4A, only supernatants from R848-treated neutrophils were capable of inducing the appearance of cord blood-derived Th17 cells (from 1 to 6 %, n=4), as determined by the induction of intracellular IL-17 associated with an increased expression of CD161 (a Th17 marker)[22]. By contrast, supernatants from either untreated neutrophils, or $CD14^+$monocytes, regardless of their treatment with R848, had no effects on Th17 induction (Fig. 4A). Similar results were observed by using neutrophil-derived supernatants harvested after 48 h of incubation with R848 (data not shown). Finally, the addition of anti-IL-23p19 neutralizing antibodies to supernatants from R848-treated neutrophils drastically reduced the frequency of cord blood-derived Th17 cells (Fig. 4B). Altogether, these data indicate that supernatants from R848-treated

neutrophils, but not $CD14^+$monocytes, are able to promote the differentiation of Th17 cells, mainly in an IL-23-dependent fashion.

Effect of endogenous and exogenous TNFα on TLR8-induced IL-23 mRNA and protein expression.

Previous work has uncovered a role for endogenous TNFα in amplifying R848-induced expression of IL-6 [11]. Therefore, to verify whether endogenous TNFα also regulates the production of IL-23, we incubated neutrophils with R848 for 20 h, in the presence or the absence of 10 μg/ml adalimumab, a TNFα neutralizing antibody [34]. Fig. 5A shows that the expression of IL-23 and IL-12B proteins, induced by R848, is largely dependent on endogenous TNFα (IL-23 and IL-12B protein decreased by 79 % and 70 %, respectively in the presence of adalimumab). Fig. 5B shows that such adalimumab-mediated inhibitory effect also occurs at the level of mRNA expression for both IL23A and IL12B (IL23A and IL12B mRNA on average decreased by 80 % and 73 %, respectively, by adalimumab). We subsequently incubated neutrophils with or without R848, in the presence or the absence of 10 ng/ml TNFα, to further investigate the direct ability of TNFα to trigger the expression/production of IL-12 family members. As shown in Fig. 5C, exogenous TNFα alone was able to trigger both IL-23 and IL-12B expression, albeit at lower levels than R848. However, the combination of R848 plus TNFα produced a synergistic/additive effect on the production/release of both IL-23 and IL-12B (Fig. 5C), and these effects were mirrored at the mRNA level (Fig. 5D). Nonetheless, in the *in vitro* model of Th17 differentiation from UCB-derived $CD4^+$ T cells, supernatants from R848 plus TNFα-treated neutrophils did not increase the frequency of IL-17 producing cells compared to supernatants derived from neutrophils treated with R848

only (Figure 4B). Once again, neither expression of IL12A mRNA, nor production of IL-12 was detectable by neutrophils incubated with R848 plus TNFα (Fig. 5C and 5D). In parallel experiments, we incubated neutrophils with or without R848, in the presence or the absence of 10 ng/ml GM-CSF. However, GM-CSF by itself was unable to trigger the expression/production of IL12A, IL12B and IL23A, and it also did not potentiate the effects of R848 on the same genes/proteins (data not shown).

To get more insights into the molecular mechanisms underlying the synergistic induction of both IL12B and IL23A mRNA transcription by R848 plus TNFα, we performed H3K27Ac and PU.1 ChIP-qPCR experiments targeting selected regulatory regions of the neutrophil IL12B (indicated by the black, white and grey boxes in Fig. 6A) and IL23A (indicated by the black and white boxes in Fig. 6C) loci. These regions were chosen on the basis of the high deposition of PU.1 and H3K27Ac observed in our ChIP-seqs (Fig. 2). As shown in Figure 6, we found that either TNFα, or (more efficiently) R848, alone triggers an increase of the levels of H3K27Ac and PU.1 at all regulatory regions of both IL12B (Fig. 6B) and IL-23A (Fig. 6D). Costimulation of neutrophils with R848 plus TNFα further raised the levels of H3K27Ac and PU.1 already induced by either R848 or TNFα alone, resulting in an additive effect.

DISCUSSION

We have recently discovered that activation of highly pure human neutrophils with TLR8 triggers the production of remarkable quantities of cytokines, including TNFα, IL-6, G-CSF and CCL23 [11, 13, 14]. In this study, we demonstrate that, under the same experimental conditions, human neutrophils also express and produce IL-23, one of the members of the IL-12 family that is generated from the association of the IL-23A/IL-23p19 and IL-12B/IL-12p40 subunits. By contrast, human neutrophils were found not to express IL-12/IL-12p70, which are derived from the association of the IL-12A/IL-12p35 and IL-12B/IL-12p40 subunits. We show that TLR8-activated neutrophils are induced to time-dependently express transcripts for IL12B (encoding IL-12p40) and IL23A (encoding IL-23p19), but not IL12A (encoding IL-12p35), as determined by RNA-seq and RT-qPCR experiments. We also show that they produce and release the corresponding proteins, IL-12B, IL-23A and IL-23, but not IL-12, as determined by ELISA. Incubation of highly pure human neutrophils with LPS (a TLR4 agonist) also induced similar patterns of expression, although the amounts of IL-12B, IL-23A and IL-23 recovered in neutrophil-derived supernatants were lower than those measured after stimulation with TLR8 agonists. In contrast, autologous $CD14^+$ monocytes incubated with either R848 or LPS were found to produce IL-23A, IL-12B and IL-23 at much higher levels than neutrophils. However, while in neutrophils R848 was a more potent trigger of IL-23 production than LPS, in monocytes both agonists had near equal potency. Moreover, $CD14^+$ monocytes could also produce IL-12, but, consistent with the literature [30, 31, 35], only if incubated with TLR8 agonists. These differences in cellular responses to different agonists clearly demonstrate that the results obtained with our highly pure neutrophil populations are not due to their potential contamination with $CD14^+$ monocytes. That neutrophils

express IL12B and IL23A, but not IL12A, mRNA, in response to either TLR8 or TLR4 activation implicate intracellular signaling cascades triggered by the MyD88-dependent pathway [36]. Signaling via this pathway leads in fact to the activation of NFκB and MAPKs, which ultimately regulate the transcription of IL-12p40, IL-23p19 [37-39]. In contrast, the induction of IL12A gene expression by TLR activation in myeloid cells is usually dependent on endogenous type I IFN [40], whose production, at least in the case of TLR4 activation results from the stimulation of the TRIF-dependent pathway [41]. For reasons that are not yet explained at the molecular level, LPS-treated neutrophils are unable to trigger the TRIF-dependent cascade and consequently do not produce type I IFN [42, 43]. Similarly, while monocytes stimulated by TLR8 agonists produce type I IFN via IRF5 activation [44], it is not known whether this pathway is somewhat defective in human neutrophils. In any case, the lack of type I IFN production would contribute to the explanation as to why human neutrophils do not express IL-12A mRNA in response to either TLR8 or TLR4 activation. Another potential explanation for the inability of neutrophils to accumulate IL-12A mRNA is that they do not express IRF8, a transcription factor that, in association with IRF1 at the *IL12A* locus, is essential for IL-12A transcription in monocytes[32].

We performed ChIP-seq experiments to characterize the H3K27Ac and PU.1 levels at the *IL12A*, *IL12B* and *IL23A* genomic loci, in order to identify their genomic regulatory regions[28]. Our experiments revealed that the *IL12B* and *IL23A* loci are in a latent state in unstimulated neutrophils, and that, upon TLR8 activation, they become not only accessible to lineage determining transcription factors, such as PU.1, but also become marked by histone modifications characteristic of active genomic regions (H3K27Ac). Therefore, as in the case of IL-6 mRNA expression[11], changes in the

chromatin landscape induced by R848 appear to regulate IL12B and IL23A mRNA induction in human neutrophils. By contrast, we found that the *IL12A* locus remained inactive regardless of neutrophil-treatment with R848, and therefore preventing IL12A mRNA transcription, consistent with our RNA-seq and RT-qPCR data. In this respect, the chromatin status of the *IL12A* locus in human neutrophils resembles that of IL-10, which we have previously shown to be in a closed/inactive conformation, preventing its transcription [45]. Moreover, because of the lack of IL12A gene expression, it is plausible to speculate that TLR4/TLR8-activated neutrophils do not even produce IL-35, which is another heterodimeric cytokine of the IL-12 family composed by the association of the IL-12A and EBI3 subunits.

In additional experiments, we incubated neutrophils with GM-CSF, alone, or in combination with R848, and found that GM-CSF neither induced the expression of *IL12A*, *IL12B* and *IL23A* mRNA, nor influenced the effects of R848 on the same genes. In contrast, the use of a TNFα neutralizing antibody, adalimumab[34], demonstrated that the production of IL-23 by TLR8-activated neutrophils is partially dependent on the endogenous expression and release of TNFα. Adalimumab decreased the production of IL-23 protein by approximately 70 %, and also decreased levels of *IL12B* and *IL23A* transcripts. The effect of adalimumab on IL-23 production is reminiscent of that on IL-6, for which endogenous TNFα was also shown to play an amplifying role [11, 14]. However, while TNFα exogenously added to neutrophils was unable by itself to trigger the production of IL-6 [11], it was able to induce IL-23 expression, albeit at much lower levels than R848. Moreover, when used in combination with R848, exogenous TNFα resulted in a synergistic effect on the production and release of IL-23 by enhancing the expression of both *IL12B* and *IL23A* transcripts. Such a different capacity of exogenous TNFα to trigger low levels of IL-

428 23, but not IL-6, mRNA expression/production, might be explained by the fact that in
429 resting human neutrophils, the chromatin at the *IL6* genomic locus is inaccessible [11],
430 while that at the *IL12B* and *IL23A* genomic loci is more opened and thus accessible to
431 low levels of transcription via signal dependent transcription factors (SDTFs), such as
432 NF-κB. Accordingly, we observed that TNFα is able to activate the regulatory
433 regions at both *IL12B* and *IL23A* genomic loci, by recruiting PU.1 and by favoring the
434 deposition of H3K27Ac. Notably, we also observed that TNFα further enhanced the
435 effects triggered by R848 at the same regions, explaining the enhanced transcription
436 at these regions in the presence of both agonists. On the other hand, the stimulation of
437 neutrophils with TNFα together with R848 did not induce either the expression of
438 IL12A mRNA or the IL-12 production, indicating that even the combination of these
439 two potent stimuli is not sufficient to provoke a chromatin remodeling at the IL12A
440 locus.

441 Since IL-23 represents one of the cytokines responsible for the differentiation
442 of Th17 cells[33], along with IL-1β, IL-6 and TGFβ1, we tested whether supernatants
443 harvested from R848-treated neutrophils could promote this process. Of note, in our
444 system of Th17 induction [22], we detected the appearance of low but reproducible
445 levels of Umbilical cord blood (UCB)-derived Th17 cells from *naïve* $CD4^+$ T cells
446 incubated for one week with supernatants from neutrophils cultured with R848, but
447 not in medium from unstimulated cells. However, we did not observe the same effects
448 on Th17 induction using supernatants from $CD14^+$ monocytes cultured with or
449 without R848. The findings that supernatants derived from neutrophils stimulated
450 with R848 plus TNFα did not increase the frequency of Th17 UCB-derived $CD4^+$ T
451 cells compared to the R848-stimulated only supernatants, despite the higher levels of
452 IL-23, might be due to the fact that they contain additional cytokines negatively

interfering with Th17 polarization. In fact, the Th17 differentiation obtained after 1 week of *in vitro* culture derives from many polarizing signals on naïve $CD4^+$ T cells and depends on the balance of different cytokines in the culture medium. Moreover, the experiments performed in the presence of anti-IL-23p19 neutralizing antibody clearly demonstrate that IL-23 present in supernatants from R848- (and R848 plus TNFα)-treated neutrophils is mainly responsible for their Th17-promoting effects, thus emphasizing previous findings on the crosstalk between neutrophils and Th17 cells [46, 47]. Accordingly, human neutrophils and Th17 cells have been previously shown to reciprocally chemoattract each other *via* the production of various chemokines, including CCL20 from neutrophils and CXCL8 from Th17 cells[48]. Moreover, GM-CSF and/or IFNγ derived from Th17 cells were shown to promote neutrophil survival as well as enhance various neutrophil effector functions [48]. More recently, neutrophil-derived elastase has been shown to process DC-derived CXCL8 into a truncated, potent Th17 cell–inducing form [49]. Our current data on the ability of TLR8-activated neutrophils to also drive *via* IL-23 the differentiation of Th17 cells, is consistent with another study showing that Group B Streptococcus (GBS)–stimulated neonatal neutrophils induce robust Th1– and Th17–type responses in neonatal $CD4^+$ T-cell and Treg populations through mechanisms involving cell–cell contact and soluble mediators [50], adding new knowledge on the regulation and activation of the neutrophil/Th17 cell crosstalk. It is now necessary to determine if such processes occur *in vivo* during inflammation or infections.

Our data greatly extend previous observations on the ability of human neutrophils to express/produce IL-23. For example, it was previously shown that, while *Helicobacter pylori* Neutrophil Activating Factor (HP-NAP) triggers the

expression of IL23p19 mRNA by human neutrophils[51], *Borrelia burgdorferi* NapA, but not OspA, induces IL-23 at both gene expression and protein level[52]. In another study, both blood neutrophils and neutrophils infiltrating colon tissue of pediatric patients with inflammatory bowel disease were found to express IL-23p19 (as revealed by immunohistochemistry and immunofluorescence)[53], using an antibody detecting only the IL-23p19 monomer, which is secreted by many cell types but is biologically inactive. More recently, human neutrophils (whose purity was not stated) were shown to express IL-23 (presumably IL-23p19) mRNA, as well as to produce very elevated levels of IL-23 protein, when infected with *Mycobacterium tuberculosis* (MTB) H37Rv or when incubated with either LPS or Pam3CSK4 (a TLR2 agonist)[54]. However, in these experiments the infected neutrophils were also reported to express IL-17[54], which, according to our previous work[55], does not occur. Finally, immunofluorescence and flow cytometry analyses revealed that, in patients with castration-resistant prostate cancer (CRPC), tumour-infiltrating myeloid-derived suppressor cells (MDSCs) with the neutrophil phenotype ($CD11b^+CD33^+CD15^+$ cells, PMN-MDSCs) express IL-23[56].

In summary, we show that highly pure neutrophils express IL-23, which is capable of driving the differentiation of Th17 cells. The expression of this key cytokine by human neutrophils requires chromatin remodeling at the normally transcriptionally silent IL-23 gene loci by agents such as TLR8 agonists. These data clearly support the role for neutrophils in the regulation of inflammation via the important IL-17/IL-23 network.

Authorship

Experimental design: NT, MAC, SWE, RJM, LM, FA.

Experimental work: NT, FAS, EG, FBA, SG, FM, MC.

Data Analysis: NT, FAS, FBA, FM, HLW, LM, FA

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Disclosures

The authors declare no conflicts of interest

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Figure Legends

Figure 1. Expression of IL12A, IL12B and IL23A mRNA in neutrophils incubated with R848. In **A**, highly pure neutrophils were incubated for 20 h in the absence (-) or the presence of 5 μM R848. Expression levels of transcripts for IL12A (white bars), IL12B (grey bars) and IL23A (black bars) were measured by RNA-seq (n = 2). Similar results were observed from RNA-seq of neutrophils incubated with R848 for 7 h (n=2, not shown). In **B**, highly pure neutrophils were incubated for up to 20 h in the presence or the absence of 5 μM R848, and then IL12A (B), IL12B (C) and IL23A (D) mRNA expression was measured by RT-qPCR. Gene expression is depicted as mean normalized expression (MNE) units after normalization to GAPDH mRNA (mean ± SEM, n = 3-13). Asterisks indicate significant differences: ** $P < 0.01$, *** $P < 0.001$, by two-way ANOVA followed by Bonferroni's post-test.

Figure 2. ChiP-seq profiles of H3K27Ac and PU.1 at the *IL12A*, *IL12B* and *IL23A* genomic loci in human neutrophils incubated with or without R848. Genomic snapshots showing PU.1 and H3K27Ac peaks, as well as mRNA expression levels for *IL12A* (**A**), *IL12B* (**B**) and *IL23A* (**C**), in neutrophils incubated for 20 h in the absence (-) or the presence of R848. Changes in the regulatory regions (boxed in **A**, **B** and **C**) of the *IL23A* and *IL12B* genes, but not the *IL12A* gene, can be observed in human neutrophils following treatment with R848.

Figure 3. Human neutrophils produce and release IL-23, but not IL-12, in response to either R848 or LPS. Highly pure neutrophils (**A**) and autologous $CD14^+$

monocytes (**B**) were incubated for 20 and 44 h in the absence (-) or the presence of either 5 μM R848 or 1 μg/ml LPS. Cell-free supernatants were then collected and the levels of IL-23, IL-12B and IL-12 proteins measured by ELISA (n = 6). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, by two-way ANOVA followed by Bonferroni's post-test. (E) Immunoblot displaying IRF8 and actin protein expression in neutrophils and autologous $CD14^+$ monocytes, either freshly isolated or cultured for up to 20 h with or without 5 μM R848 (representative experiment, n = 2).

Figure 4. Biological activity of neutrophil-derived supernatants

Umbilical cord blood (UCB)-derived $CD4^+$ T cells were incubated for 1 week with 5 μg/ml of anti-CD3 plus 5 μg/ml anti-CD28 mAbs, **(A)** in the absence or the presence of 10 ng/ml IL-1β plus 20 ng/ml IL-23, or the indicated conditioned supernatants derived from neutrophils or $CD14^+$ monocytes incubated with or without R848 for 20 h; (**B**) in the absence or the presence of 10 ng/ml IL-1β plus 20 ng/ml IL-23, or the indicated conditioned supernatants derived from neutrophils incubated with or without R848, or R848 plus TNFα for 20 h and used in culture in the presence or absence of 10 μg/ml neutralizing human anti-IL-23p19 antibody. Intracellular IL-17 production and CD161 expression were evaluated by flow cytometry after stimulation with PMA plus ionomycin (see M&M). Representative experiments out of four and two are shown in panels A and B, respectively.

Figure 5. R848-induced production of IL-23 by neutrophils is partially dependent on, and amplified by, TNFα. Highly pure neutrophils **(A**,**B)** were pre-

incubated for 30 min with 10 μg/ml adalimumab and then cultured for 20 h in the presence of either 5 μM R848 or 10 ng/ml TNFα. In **(C**,**D)**, neutrophils were incubated for 20 h in the absence (-) or the presence of R848, 10 ng/ml TNFα, or both agonists in combination. The levels of IL-23 (**A, C**), IL-12B (**A, C**) and IL-12 (**C**) proteins were measured by ELISA (n = 6), while (**B**) IL23A (**B**, **D**), IL12B (**B**, **D**) and IL12A (**D**) mRNA expression was determined by RT-qPCR (n = 6). Gene expression is depicted as mean normalized expression (MNE) units after normalization with GAPDH mRNA (mean ± SEM). ** $P < 0.01$, *** $P < 0.001$, by **(A, B)** one-way ANOVA followed by Tukey's post-test or **(C, D)** two-way ANOVA followed by Bonferroni's post-test.

Figure 6. H3K27Ac and PU.1 levels at the *IL12B* and *IL23A* genomic loci of neutrophils incubated with R848, alone or in combination with TNFα.

(**A**, **C**) Schemes illustrating the positions of the designed primer pairs amplifying promoter and potential enhancer regions of IL12B (indicated by the black, white and grey boxes) and IL23A (indicated by the black and white boxes) for ChIP analysis. Panels **B** and **D** show the enrichment levels of H3K27Ac (left panels) and PU.1 (right panels) at the *IL12B* (**A**) and *IL23A* (**C**) genomic loci by chromatin immunoprecipitation (ChIP) analysis in human neutrophils incubated for 20 h with or without 5 μM R848 and/or 10 ng/ml TNFα. Coimmunoprecipitated DNA samples were expressed as percent of the total input. Panels in **B** and **D** depict a representative experiment out of three independent ones with similar results. Error bars represent SE calculated from triplicate qPCR reactions.

Table 1: List of human primer sets utilized for the RT-qPCR experiments.

RT-qPCR primers	Sequences	
	forward primers	reverse primers
IL12A	CTGGACCACCTCAGTTTGG	TTTGTCGGCCTTCTGGAG
IL12B	GGACATCATCAAACCTGACC	AGGGAGAAGTAGGAATGTGG
IL23A	GGACACATGGATCTAAGAGAAGAG	CTATCAGGGAGCAGAGAAGG
RPL32	AGGGTTCGTAGAAGATTCAAGG	GGAAACATTGTGAGCGATCTC

Table 2: List of human primer sets utilized for qPCR of ChIP assay.

ChIP primer name	Location (relative to the gene TSS)	Sequence	
		forward primers	reverse primers
IL12B #1	-308 to -64	CCCTCCTCGTTATTGATACACAC	GCTTGGGAAGTGCTTACCTTG
IL12B #2	-12163 to -12070	GCAGAGGCAACACCTAAAGC	GCCCTTGATGAAGAAATGAGTG
IL12B #3	-29315 to -29125	CCACTTCCCTTTTGACTTTAGG	CCCTGGGTTAGTACAGATTCG
IL23A #1	-2166 to -2055	AGTTGTAGCCCTGGATGTAGTTC	CTCTGCCTCTTTGTTTCACTTC
IL23A #2	-5233 to -5072	GATAGGGCAAGGGTCAGATG	GGAGAACTGGGGAAACTGG
PRL	+386 to +506	AGGGAAACGAATGCCTGATT	GCAGGAAACACACTTCACCA

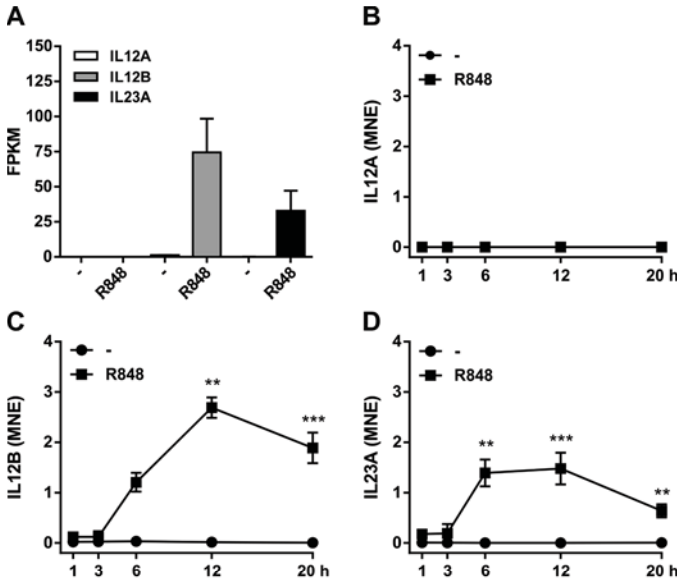
A
150
125
100
75
50
25
0
FPKM
IL12A
IL12B
IL23A
-
R848
-
R848
-
R848
B
4
3
2
1
0
IL12A (MNE)
-
R848
1
3
6
12
20 h
C
IL12B (MNE)
-
R848
**
***
1
3
6
12
20 h
D
IL23A (MNE)
-
R848
**
***
**
1
3
6
12
20 h

Figure 1

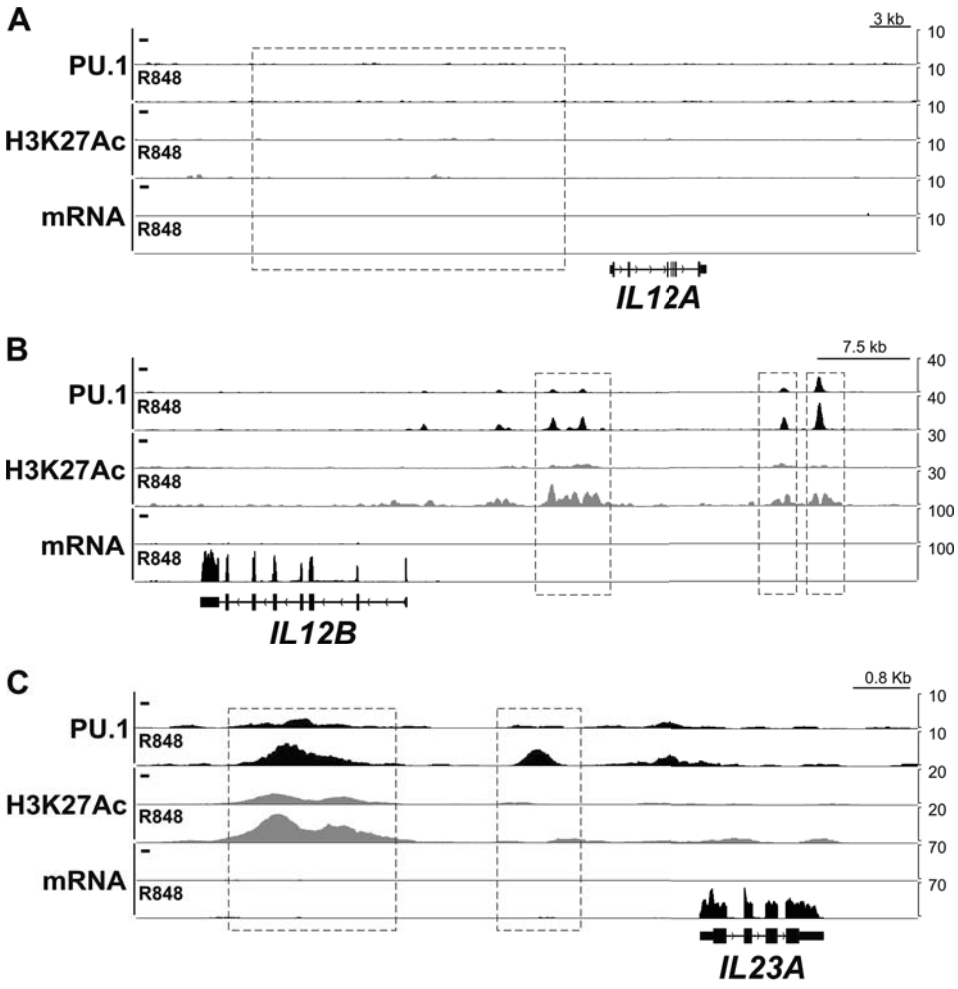
A
3 kb
PU.1
H3K27Ac
mRNA
–
R848
10
10
10
10
10
10
IL12A
B
7.5 kb
PU.1
H3K27Ac
mRNA
40
40
30
30
100
100
IL12B
C
0.8 Kb
PU.1
H3K27Ac
mRNA
10
10
20
20
70
70
IL23A

Figure 2

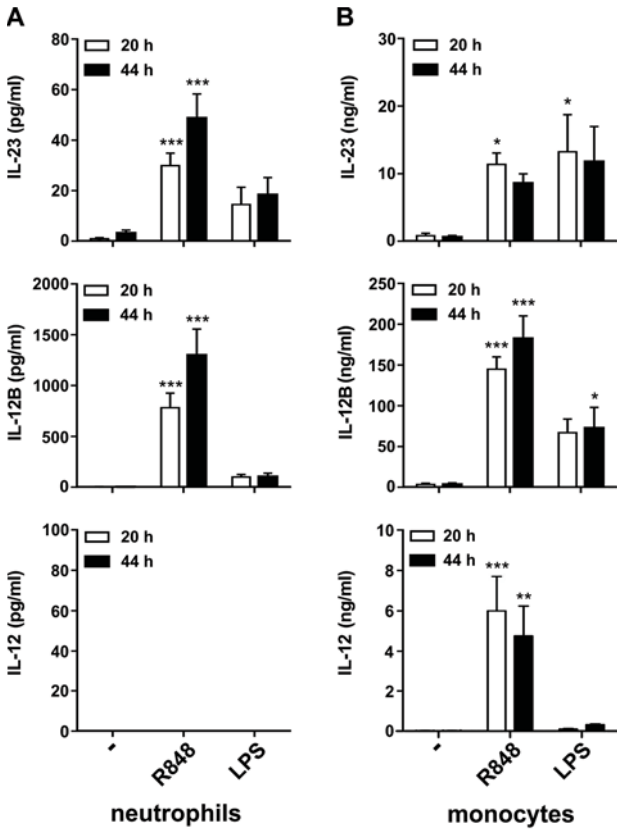
A
B
20 h
44 h
IL-23 (pg/ml)
IL-23 (ng/ml)
IL-12B (pg/ml)
IL-12B (ng/ml)
IL-12 (pg/ml)
IL-12 (ng/ml)
-
R848
LPS
neutrophils
monocytes
C
neutrophils
monocytes
(kDa)
49
37
IRF8
actin
T0
-
R848
20 h

Figure 3

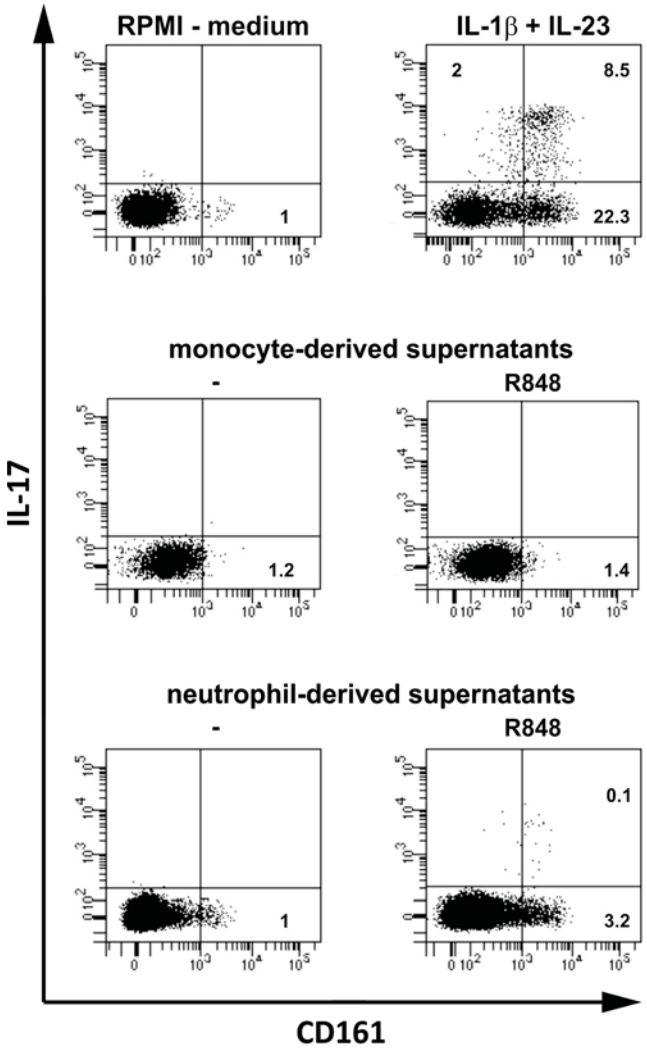
RPMI - medium
IL-1β + IL-23
2
8.5
1
22.3
monocyte-derived supernatants
-
R848
1.2
1.4
neutrophil-derived supernatants
-
R848
0.1
1
3.2
IL-17
CD161

Figure 4A

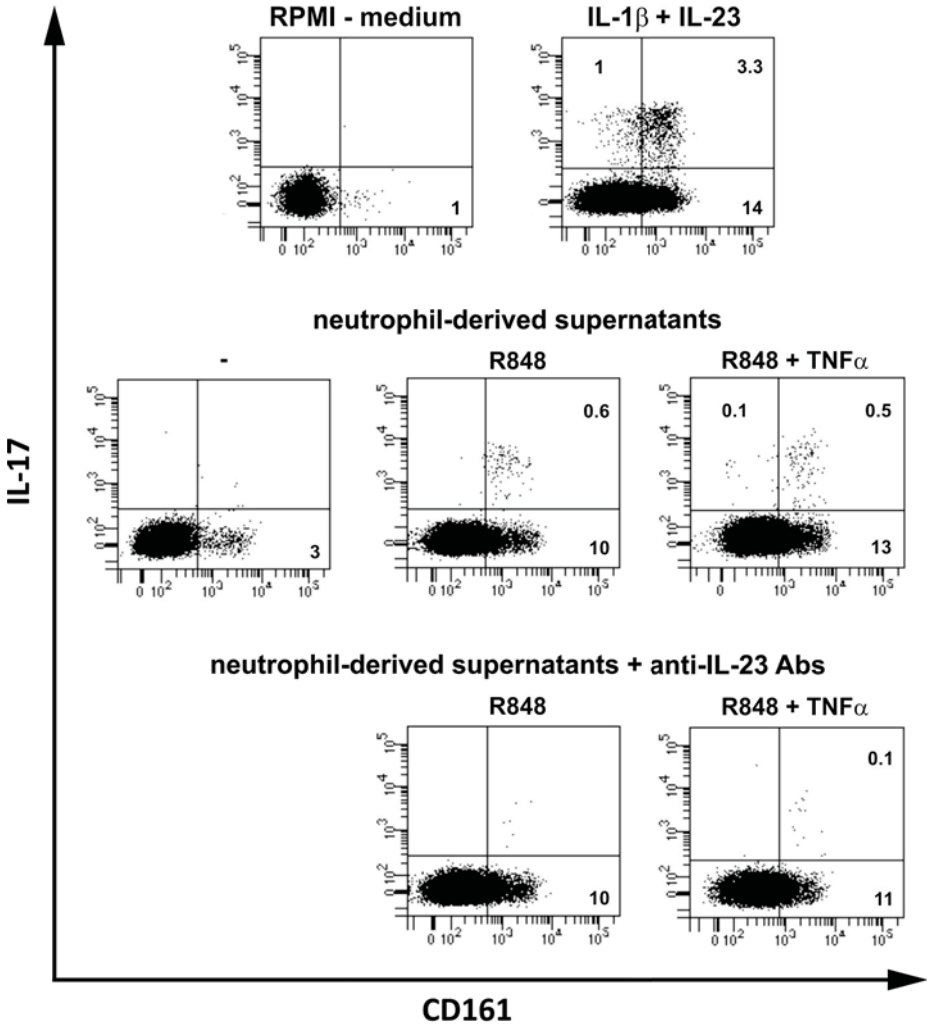
RPMI - medium
IL-1β + IL-23
1
3.3
1
14
neutrophil-derived supernatants
-
R848
R848 + TNFα
0.6
0.1
0.5
3
10
13
neutrophil-derived supernatants + anti-IL-23 Abs
R848
R848 + TNFα
0.1
10
11
IL-17
CD161

Figure 4B

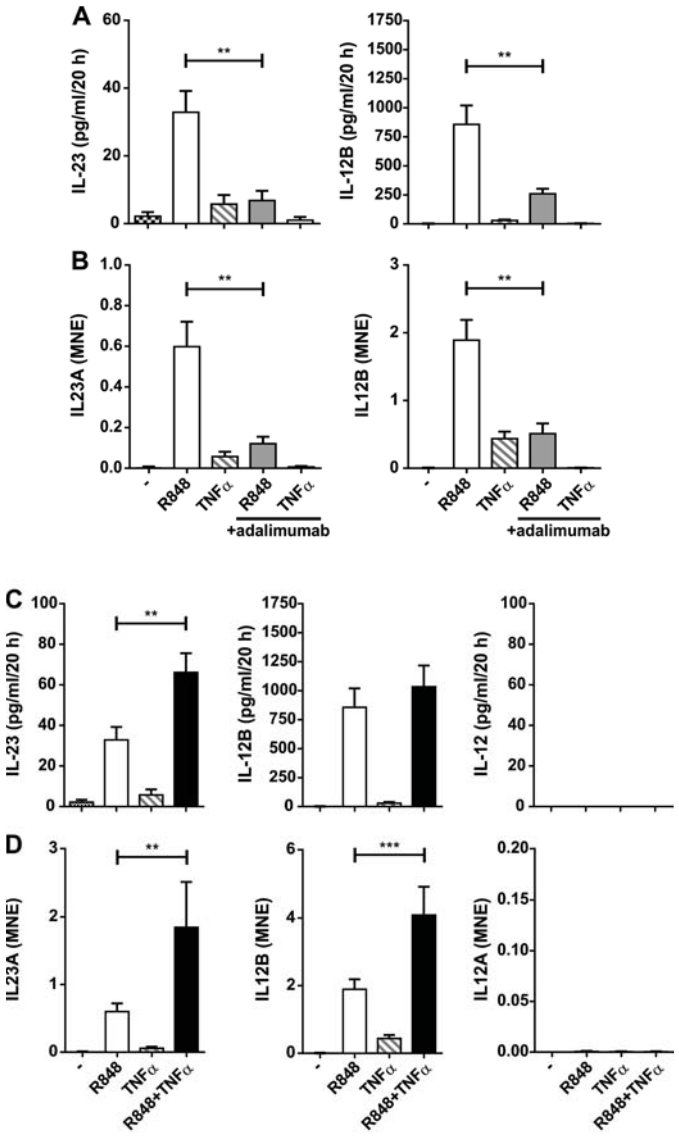
A
IL-23 (pg/ml/20 h)
IL-12B (pg/ml/20 h)
B
IL23A (MNE)
IL12B (MNE)
-
R848
TNFα
R848
TNFα
+adalimumab
C
IL-23 (pg/ml/20 h)
IL-12B (pg/ml/20 h)
IL-12 (pg/ml/20 h)
D
IL23A (MNE)
IL12B (MNE)
IL12A (MNE)
-
R848
TNFα
R848+TNFα

Figure 5

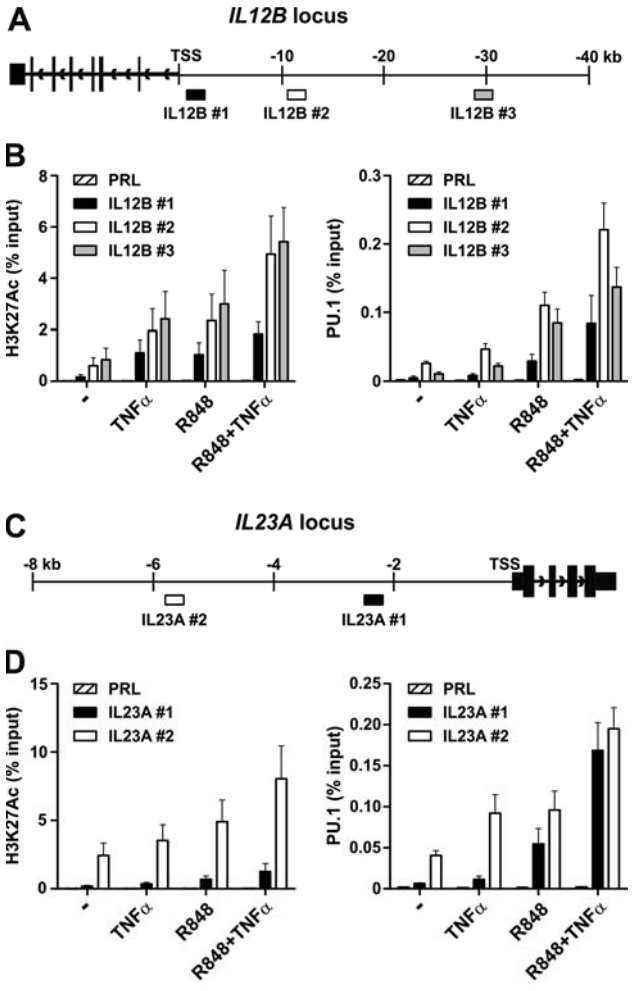
A
IL12B locus
TSS
-10
-20
-30
-40 kb
IL12B #1
IL12B #2
IL12B #3
B
H3K27Ac (% input)
PU.1 (% input)
PRL
IL12B #1
IL12B #2
IL12B #3
-
TNFα
R848
R848+TNFα
C
IL23A locus
-8 kb
-6
-4
-2
TSS
IL23A #2
IL23A #1
D
H3K27Ac (% input)
PU.1 (% input)
PRL
IL23A #1
IL23A #2
-
TNFα
R848
R848+TNFα

Figure 6